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Establishment of Reference Methods for Lipids, Lipoproteins and Apolipoproteins[1])

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Summary: Reference methods for lipids, lipoproteins, and apolipoproteins have been developed for use as part of an accuracy base for institutional, national, or international reference systems. A widely accepted reference method exists only for total cholesterol. Well described interim or institutional in-house reference methods have been established for the other lipids, lipoproteins, and apolipoproteins. The major criteria for a reference method are

1) scientific basis,

2) sound principles,

3) available calibration and control materials,

4) traceability to a definitive method or a point of reference, and

5) applicability to reference materials that provide traceability to clinical methods and transferability to other reference laboratories.

The total cholesterol reference method of the U. S. National Reference System demonstrates how a reference method can be developed and applied. Reference methods now available can lead to an accepted international accuracy base for the clinically useful lipid, lipoprotein, and apolipoprotein measurements.

Introduction

Reference methods are essential components of the accuracy base of any established reference system for lipids, lipoproteins, and apolipoproteins. A stable accuracy base depends upon availability of a stable reference method and reference materials. Together, a reference method and reference material play a significant role in transferring the accuracy base of a definitive method to the clinical laboratory. Reference methods are considered acceptable when they possess

1) known minimum bias versus a definitive method,

2) known sound scientific basis,

3) available calibration and quality control reference material, and

4) traceability to clinical methods and their secondary serum reference material.

The more widely available reference methods designed for widespread use and application must be validated by the less available definitive method (1). A purified

[1]) Contribution of the International Federation of Clinical Chemistry (IFCC) Working Group on Apolipoprotein Reference Methods established at the Centers for Disease Control and the IFCC Committee on Apolipoproteins (*S. M. Marçovina*, chairman, *J. J. Albers, J. C. Fruchart, M. Rosseneu, J. Shepherd*) of the IFCC Scientific Division (*H. Greiling*, chairman, *M. M. Müller*, vice chairman).
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primary standard is required to calibrate the definitive method and reference method while commutable secondary serum reference materials are used to calibrate clinical methods of lipids, lipoproteins, and apolipoproteins. A higher productivity or throughput than the definitive method provides is desirable to satisfy multiple demands placed upon a reference method.

Reference methods are developed to assure accuracy of clinically useful laboratory procedures. Procedures for lipid, lipoprotein, and apolipoprotein considered highly useful to the clinicians in the detection, prevention, and control of coronary heart disease and secondary hyperlipidaemia diseases are quantitative measurements of total cholesterol, triacylglycerol, high-density lipoprotein (HDL)[2]) and low-density lipoprotein (LDL), cholesterol, and apolipoproteins A-1 and B. These therefore will be discussed here.

Reference methods have been established at the Centers for Disease Control (CDC) for total cholesterol, triacylglycerol, HDL cholesterol, and apolipoprotein A-1 and B (2). The total cholesterol method has been accepted as the total cholesterol reference method of the US National Reference System for Cholesterol established by the National Committee for Clinical Laboratory Standards (NCCLS) (3). The US National Reference System for Cholesterol comprises the isotope dilution and mass spectrometric definitive method of the National Institute of Standards and Technology (NIST) (4), CDC modified *Abell—Levy—Brodie—Kendall* (5) total cholesterol reference method (6, 7), NIST-certified pure cholesterol standard, and NIST- and CDC-certified serum based secondary reference material (8). The NCCLS has accepted the CDC in-house reference methods for triacylglycerol and HDL cholesterol as NCCLS interim reference methodological principles for the U. S. National Reference System. CDC and the Committee on Apolipoproteins of the International Federation of Clinical Chemistry (IFCC) are collaborating in studies designed to select candidate reference methods for apolipoproteins A-1 and B.

The total cholesterol reference method will be discussed in greater detail than those of the other lipids, in order to illustrate the characteristics, requisites, and applications of a reference method and its role in standardization.

[2]) Non-standard abbreviations:
CDC Centers for Disease Control
NIST National Institute for Standards and Technology
SRM Standard Reference Material
RIA radioimmunoassay
VLDL very low-density lipoprotein
IDL intermediate-density lipoprotein
LDL low-density lipoprotein
HDL high-density lipoprotein

Essential Characteristics of a Reference Method

The major criteria for a reference method are

1) scientific basis,
2) sound principles,
3) available calibration and control materials,
4) traceability to a definitive method or a point of reference,
5) applicability to reference materials that provide traceability to clinical methods, and
6) transferability to other reference laboratories (9—12).

The scientific basis is judged by accuracy, precison, linearity of dose-response, specificity, stability of reagents, interferences, recovery, and ruggedness. Sound principles demand that each analytical step must be defined and controlled, possess appropriate sensitivity, permit accurate sampling and dilutions, use stable equipment, calculate results by accepted procedures, and be subject to acceptable analysis of variance. Each reference method should use a defined purified primary standard or defined serum biological reference preparation for use in calibration or monitoring. The serum reference materials should have minimum matrix effects, be stable over time, and permit valid assignment of mass values. Directions for the reference method should be written so clearly that the reference method can be transferred to other laboratories without any instructions other than the directions. These essential characteristics form the criteria that a method must meet to be considered as a candidate reference method for the measurement of lipids, lipoproteins, and apolipoproteins in serum or other body fluids.

Total Cholesterol Reference Method

In 1975, the Cholesterol Reference Method Study Group of the American Association for Clinical Chemistry (AACC) invited the CDC to develop a candidate total cholesterol reference method. During the five-year development period (1977—1982), five potential candidate methods were selected, optimized, and compared. The top two methods (*Abell—Levy—Brodie—Kendall* (5) and an enzymic procedure) were selected on the basis of these studies, and were then compared with the NIST-developed isotope dilution-mass spectrometric definitive method (4). On the basis of these comparisons, the American Association for Clinical Chemistry Cholesterol Reference Method Study Group selected the *Abell—Levy—Brodie—Ken-*

dall method as the recommended total cholesterol reference method (5). The *Abell—Levy—Brodie—Kendall* method was next subjected to extensive validation studies (6) and a transferability study with 14 collaborating lipid laboratories throughout the United States (7). The transferability study demonstrated that the recommended reference method permits laboratories to attain a coefficient of variation (CV) of less than 1.5% within the laboratory and CV of less than 3.0% among the 14 laboratories. The mean percent bias value of the 14 laboratories compared with that of the CDC was less than 1% for six of the 14 laboratories, less than 1.5% for 12, and less than 3% for all 14 laboratories (7). Interestingly, a preliminary readiness testing and competence evaluation predicted the quality of analytical performance exhibited during the transferability testing.

The total cholesterol reference method procedure includes the following steps: a 0.5-ml sample in 20 g/l alcoholic KOH undergoes hydrolysis at 50 °C for 1 h; the hydrolysate is extracted with hexane by vigorous shaking at 25 °C for 15 min; an aliquot of hexane is evaporated in a vacuum oven at 55 °C; colour is developed by reaction with a reagent formed from 20 parts acetic anhydride, 1 part H_2SO_4, and 10 parts glacial acetic acid at 25 °C for exactly 30 min; and an absorbance reading is taken at 620 nm in a flow-through cuvette.

Calibration is performed with NIST pure cholesterol standard reference material 911b (6). Since 1978, analytical performance of the total cholesterol reference method at CDC has exhibited a CV of less than 1% and a bias rarely above 1% on frozen serum pools (tab. 1).

Tab. 1. Performance of total cholesterol reference method, CDC 1989

	October	November	Cumulative
Expected value = 179.8 mg/dl (4.65 mmol/l)			
N	32	22	190
Mean	180.2 (4.66)	180.5 (4.67)	180.0 (4.65)
Bias (%)	+0.18	+0.37	+0.09
CV (%)	0.58	0.59	1.53
Expected value = 276.2 mg/dl (7.14 mmol/l)			
N	31	18	183
Mean	277.0 (7.16)	247.7 (7.10)	275.2 (7.12)
Bias (%)	+0.30	−0.53	−0.38
CV (%)	0.64	1.10	1.07
Expected value = 298.6 mg/dl (7.72 mmol/l)			
N	12	8	239
Mean	300.8 (7.78)	301.6 (7.80)	298.9 (7.73)
Bias (%)	+0.71	+1.09	+0.97
CV (%)	0.38	0.43	0.97

CDC and NIST have collaborated since 1978 to maintain a defined accuracy base with the CDC reference method and the NIST definitive method. Five frozen serum pools were used originally to compare the reference method with the definitive method. The agreement between the two methods was considered excellent with a difference of less than 1.0% of all five pools (2). In 1989, comparisons of the reference method and definitive method revealed excellent agreement (0.41%) on standard solutions (SRM 911b) and good agreement (1.6%) on fresh, frozen, or lyophilized sera (13). Unpublished experimental studies at CDC on potential interferences in the reference method indicate that this difference in level of results between the reference method and definitive method results largely from sterols in serum (14).

Since 1961, the CDC total cholesterol reference method has served as a part of the accuracy base for the CDC-National Heart, Lung, and Blood Institute Lipid Standardization Programme. This accuracy base has been transferred through frozen serum reference materials distributed to the several hundred U.S. participants, as well as to many international laboratories. The reliability of the reference method has made it possible to assure the accuracy of laboratories supporting more than 30 cardiovascular epidemiologic or clinical investigations coordinated by CDC-National Heart, Lung, and Blood Institute, such as the Coronary Drug Project (15), the Lipid Research Clinics (16), Multiple Risk Factor Intervention Trial (17), and the Hypertension Detection and Follow-up Study (18).

The NCCLS's National Reference System for the Clinical Laboratory Council has adopted the CDC reference method as its national reference method for cholesterol (19). The National Reference System for the Clinical Laboratory Council has designated the NIST definitive method, the CDC reference method, the NIST certified pure cholesterol SRM 911, and the CDC-certified serum-based secondary reference materials as the components of the National Reference System for Cholesterol (3).

The National Reference Method Laboratory Network has been formed by CDC to provide resources for accurate calibration of manufacturers' total cholesterol analytical diagnostic products and accurate confirmatory analysis for clinical laboratories (2). Calibration with fresh specimens has been necessary for some of the new total cholesterol reagent analytical systems that exhibit matrix effects with liquid, frozen, or lyophilized reference materials. The protocol used by the Network utilizes the NCCLS recommendation on procedure for comparison of methods using fresh

patient specimens (20). Currently, the network includes nine laboratories in the United States and one in the Netherlands: University of Minnesota, Baylor College of Medicine, Wisconsin State Laboratory of Hygiene, Cleveland Clinic Foundation, New York State Department of Health, Northwest Lipid Research Center in Seattle, Pennsylvania State Department of Health, Washington University in St. Louis, USDA Nutrition Research Center on Aging at Tufts University, and Rotterdam Academic Hospital. Network laboratories must document a CV of less than 2% and a bias of less than 1.5% at 3-month intervals in the CDC-National Heart, Lung, and Blood Institute Lipid Standardization Program.

Interim Triacylglycerol Reference Method

An in-house triacylglycerol reference method was established at CDC in 1963 on the basis of the method of *Carlson* (21, 22) and the techniques of *Van Handel & Zilversmit* (23) and *Lofland* (24). The in-house method involves silicic acid-chloroform extraction for 1 h at 25 °C, evaporation of an aliquot in a vacuum oven at 50 °C, chemical hydrolysis with 2.5 g/l alcoholic KOH for 30 min at 70 °C, allowing the solution to stand overnight at 4 °C after neutralization with 0.1 mol/l H_2SO_4, colour development by metaperiodate-arsenite-chromotropic acid reagent at 95 °C, and reading of the cooled solution in flow cell at 570 nm. The specificity of this method is largely due to the chloroform-silicic acid extraction procedure that removes free glycerol and retains minimally monoacylglycerol and diacylglycerol. The primary standard can be either pure triolein, pure palmitin, or a 2:1 mixture, respectively. Glycerol or mannitol can be used as a primary standard in calibrating or monitoring the last step in the method that analyzes the free glycerol. Laboratories' findings can lack comparability if different primary standards are used, unless results are expressed in mmol/l. True bias is not known, because a definitive method is not available. The attainable precision usually ranges between 3% and 5% CV (tab. 2).

Tab. 2. Triacylglycerol reference method performance, CDC 1989

	October	November	Cumulative
N	20	12	364
Expected value = 0.907 mmol/l			
Mean	0.918	0.910	0.917
Bias (%)	+1.20	+0.33	+1.13
CV (%)	4.0	2.2	5.6
Expected value = 2.381 mmol/l			
Mean	2.39	2.35	2.36
Bias (%)	+0.49	−1.41	−0.86
CV (%)	3.9	2.7	3.0

The major problems with the use of this triacylglycerol method as a reference method at CDC are

1) extraction with carcinogenic $CHCl_3$,
2) difficulty arising from the presence of silicic acid "fines" or very small particles in extracts,
3) impurity, instability and a potentially different degree of hydrolysis of different triacylglycerols,
4) unknown commutability of lyophilized reference material, and
5) questionable transferability of this chromotropic acid method.

Investigators are searching for a triacylglycerol reference method that may circumvent these problems.

This CDC in-house triacylglycerol reference method has been designated as the interim triacylglycerol candidate reference method for the National Reference System and serves as the selected reference method for the CDC-maintained accuracy base for the CDC-National Heart, Lung, and Blood Institute Lipid Standardization Programme and for the dedicated laboratories of National Heart, Lung, and Blood Institute clinical and epidemiologic cardiovascular investigations and trials.

Interim HDL Cholesterol Reference Method

A CDC in-house HDL cholesterol reference method combines removal of very low density lipoprotein cholesterol (VLDL) by the Beta Quan ultracentrifugal procedure (25), isolation of HDL cholesterol by precipitation of LDL cholesterol from the 1.006 kg/l Beta Quan bottom fraction by 46 mmol/l manganese and 1.3 g/l heparin reagent (25), and cholesterol analysis of the HDL cholesterol supernate by the National Reference System for Cholesterol total cholesterol reference method (6, 7). The primary standard is NIST cholesterol SRM 911 (8), and it must cover the low range of serum HDL cholesterol values, which is different from the range of serum total cholesterol. HDL cholesterol measurements are monitored with both pure cholesterol and frozen, fresh serum secondary standards. Precision for the CDC in-house HDL cholesterol reference method on low cholesterol primary standards is between 1% and 2% CV, and on frozen serum secondary reference materials, it is between 2.5% and 4% (tab. 3). True bias is not known because a definitive method is not available for HDL cholesterol measurements.

Tab. 3. HDL cholesterol reference method performance, CDC 1989

Pool prepared	HDL cholesterol mean concentration			Total CV %
	N	mg/dl	mmol/l	
7–84	470	47.0	1.22	3.0
2–87	149	39.0	1.01	3.3
Low cholesterol mean concentration				
3–85	488	51.1	1.32	1.8
8–87	72	52.5	1.36	1.6

This HDL cholesterol method has served as the reference method for the CDC-National Heart, Lung, and Blood Institute epidemiological and clinical investigations (25).

The HDL cholesterol method is also used as part of the CDC interim reference methods for VLDL and LDL cholesterol. The CDC interim reference method values for VLDL cholesterol are calculated from serum total cholesterol minus Beta Quan bottom fraction cholesterol determined by the National Reference System for Cholesterol cholesterol reference method. The CDC interim LDL reference method values are calculated from Beta Quan bottom fraction cholesterol minus HDL cholesterol determined by National Reference System for Cholesterol total cholesterol method. This interim LDL reference method determines not only the LDL, but also intermediate-density lipoproteins (IDL) and Lp(a) constituents as measured in *Friedewald* procedure (26) or in the 1.006–1.063 kg/l ultracentrifugal fractions (25).

The major problems with the interim HDL cholesterol reference method are that it has

1) no definitive method,
2) no accepted primary standard, and
3) questionable traceability to clinical and research laboratory methods that possess matrix effects.

CDC In-House Apolipoprotein B Radioimmunoassay Method

A competitive radioimmunoassay (RIA) method has been selected and is being evaluated as an interim apolipoprotein B reference method for use in the International Apolipoprotein Standardization Programme conducted by CDC. To conduct this immunoassay, a 1 : 100 dilution of sample is incubated with goat anti-LDL antibody at 37 °C for 1 h in the presence of cholate and bovine serum albumin; iodinated LDL is added, and the solution is further incubated at 4 °C for 16 h; donkey anti-anti-LDL second antibody is added, and the solution is incubated at 4 °C for 3 h in the presence of polyethyleneglycol; centrifugation is conducted at 2500 min^{-1} for 20 min; the supernant is decanted with blotting; and radioactivity of precipitate is counted. The primary standard is an LDL solution ultracentrifugally prepared at 1.030–1.050 kg/l (27). The results are calculated from bound/free ratio counts per minute versus log concentration within the range of 0.25–2.00 g/l of apolipoprotein B. This method has an intrassay variability of about 2% CV and an interassay of less than 6% CV. True bias is not known since a definitive method is not available for apolipoprotein B measurements.

Major problems exist inherently in any potential immunological reference method for apolipoprotein B measurements. Immunoassays require sensitivity and specificity of antisera, highly accurate dilutions, linear and parallel dose-response curves, and reproducible preparations of primary standards. Reported among-laboratory variation within the methods can have an average CV of between 20% and 25% for apolipoprotein B (28). The proposed (at 1.030–1.050 kg/l ultracentrifugally prepared) LDL primary standard provides an LDL preparation with decreased IDL and Lp(a) but shows limited immunological stability and uncertainty in protein analysis (29). Tracing an apolipoprotein B reference method to clinical and research apolipoprotein B immunochemical methods is difficult because of large matrix effects of reference materials in certain methods (28, 30).

During 1990, the IFCC Committee on Apolipoproteins and manufacturers of apolipoprotein diagnostic products are evaluating potential stabilized liquid serum reference materials for use as apolipoprotein B secondary serum standards. These materials, along with frozen serum pools stored at −70 °C, will be used in standardization programmes of manufacturers' apolipoprotein B diagnostic products. Difficulties and current recommendations on the standardization of apolipoprotein B measurements have been thoroughly discussed in recent publications (31–33).

CDC In-House Apolipoprotein A-1 Reference Method

A competitive RIA method, developed for use as a CDC in-house reference method for apolipoprotein A-1, is similar to the method developed for apolipoprotein B. The apolipoprotein A-1 RIA method utilizes goat polyclonal anti-apolipoprotein A-1 antiserum, and solid phase lactoperoxidase for the iodination of purified apolipoprotein A-1. The apolipoprotein A-1 primary standard is a freshly prepared, liquid, delipidated, purified preparation, confirmed for purity by amino acid analysis. The standard dis-

placement curve ranges between 0.50 and 2.0 g/l where serum samples are diluted 1 : 100 in one step. Precision measurements give an intra-assay CV of about 2%, and an inter-assay CV of less than 6%. Tracing clinical methods to a reference method for apolipoprotein A-1 should not be difficult since among-method source of variation is negligible for apolipoprotein A-1 measurements (28).

A lyophilized, purified, and delipidated proposed apolipoprotein A-1 primary standard is currently being evaluated by the European Cummunity Bureau of Reference in Brussels and the IFCC Committee on Apolipoproteins. Manufacturers of apolipoprotein diagnostic products and this IFCC committee are collaborating to develop a commutable lyophilized apolipoprotein A-1 serum secondary standard. This project seeks to gain comparability of results among commercial apolipoprotein A-1 diagnostic products. The CDC is supporting this effort by analysing these reference materials with the RIA method and studying potential primary standards and their problems.

Conclusions

A reference method has been developed for total cholesterol for national and international reference systems. Interim reference methods are being used for triacylglycerol, HDL cholesterol and LDL cholesterol measurements. Potential candidate reference methods are being developed and evaluated for apolipoprotein A-1 and B measurements by different apolipoprotein investigators. Continuing worldwide efforts to improve the methodology should lead to an accepted international accuracy base for the clinically useful lipid, lipoprotein, and apolipoprotein measurements.

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